IDENTIFICATION OF PROCYANIDIN A2 IN GRAPE AND WINE OF *VITIS VINIFERA* L. CV. MERLOT NOIR AND CABERNET SAUVIGNON

IDENTIFICATION DU PROCYANIDOLE A2 DANS LE RAISIN ET LE VIN DE *VITIS VINIFERA* L. CV. MERLOT NOIR ET CABERNET SAUVIGNON.

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Abstract: Procyanidin A2 was identified in grapes and wines of Cabernet Sauvignon and Merlot noir. But the quantification show a limited presence of this compounds in wine, near the mg/L. In this concentration the procyanidin A2 was not able to participate to the bitterness character of red wines.

Résumé : En utilisant différentes méthodes, nous avons identifié le procyanidole A2 dans des extraits partiellement purifiés de pellicules, de pépins et de vins de Merlot noir et de Cabernet Sauvignon. La particularité de ce procyanidole est de posséder outre une liaison C4-C8, une seconde liaison éther C2-O-C7. Dans la littérature de nombreux auteurs ont attribué aux formes condensées des procyanidoles, comportant une ou plusieurs liaisons de ce type, de l'amertume. Il apparaît malgré une présence constante, une faible teneur de ce procyanidole dimère, voisin du mg/L dans les vins. À ces valeurs, le procyanidole A2 ne peut pas participer au caractère amer des vins rouges.

Key words: grapes, wines, procyanidins A2, identification, quantification, bitterness

Mots clés : raisins, vins, procyanidoles A2, identification, quantification, amertume

INTRODUCTION

Proanthocyanidins represent the major part of the total polyphenol extract in hydroalcoolic solutions (RIBÉREAU-GAYON, 1969; VIVAS *et al*., 1994; FREITAS *et al*., 1996; VIVAS *et al*., 1996b). Structural units of these condensed tannins are flavan-3-ols: (+)-catechin, (-)-epicatechin for procyanidins of seeds and wines and (+)-gallocatechin, (-)-epigallocatechin for prodelphinidins of skins and wines (PRIEUR *et al.*, 1994; SOUQUET *et al*., 1996; MOUTOUNET *et al*., 1996). The most common and well known class of proanthocyanidins is the B-series corresponding to a linkage in the C4-C6 or the C4-C8 position (FLETCHER *et al*., 1977). The second class, less studied, is the A-series, which corresponds to a linkage in the C4-C8 position with an additional C2-O-C7 ether linkage (JACQUES *et al*., 1974). For comparison, the A2 **1** and B2 **2** structures were represented in the figure 1. B2 was chosen as a model on account this similarity of it's structural units (epicatechin-(4b-8)-epicatechin) with A2. Concerning the presence of this last series in grapes and wines, only SALAGOÏTY and BERTRAND (1984) present the identification of a chromatographic peak by comparison of retention time with a reference considered as a pure A2 compound. Thus occurrence of the A-series procyanidins in grapes and wines requires a new investigation.

On the other hand, for several authors, like ROOYEN and REDELINGHUYS (1983), procyanidin A2 is a bitter substance with a very low threshold value, near 2 mg/L in water. In grapes, other fruits and in wine the astringency substances are well known (HASLAM AND LILLEY, 1988; TANAKA *et al.*, 1994) but the phenolic compounds responsible of bitterness are actually unknown.

The aim of this preliminary investigation was to confirm the presence of procyanidins A2 in grapes and wines, to quantify them and to evaluate the potential participation of their structure in the bitter taste of wine.

MATERIALS AND METHODS

I - ORIGIN OF GRAPES AND WINE SAMPLES

The grape varieties are selected for their importance in the Bordeaux vineyard. For Merlot noir and Cabernet

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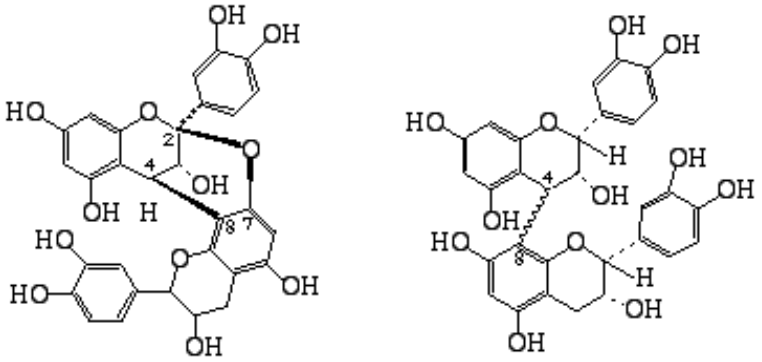

1 **2**

Figure 1 - Representation of A2 1 and B2 2 structures
Figure 1 - Représentation des structures A2 1 et B2 2

Sauvignon we selectionned different soils: Pessac, Léognan, Médoc, Haut-Médoc (Pauillac), Pomerol and Entre-Deux-Mers. Parcels were chosen for the similarity of the vine ages (35 years ± 5), the same density of plantation (5 500 plants per ha), a comparable production (for Merlot noir near 45 hl/ha and for Cabernet sauvignon near 50 hl/ha) and similar viticultural techniques. In this study we analyse only the grapes the day of harvest. The wines of these different soils and of the two varieties were vinified separately in 50 hl stainless steel tanks, with the same vinification conditions. Analysis were done 15 days after the end of malolactic fermentation.

II - ISOLATION AND CHARACTERISATION OF REFERENCE PROCYANIDINS

The pure procyanidin A2 was provided from horse chestnut shells (*Aesculus hyppocastanum*) in the conditions described by VIVAS *et al.* (1996a). Other procyanidins of B series including B1-B8 dimers and some trimers were provided by FREITAS (University of Porto, Portugal).

III - EXTRACTION AND PURIFICATION OF PROCYANIDIN FRACTION FROM RED GRAPE VARIETIES AND CORRESPONDING WINES

Skins and seeds have been separated from the pulps. They were weighed, freeze-dried, reduced to powder and then shaked separately for two minutes in a blender with 100 mL of hydroalcoholic solution (Ethanol 120 mL/L, pH 3.2). Medium was agitated during 6 h then centrifuged for 20 min. (x 4000 g). Supernatant was filtered to eliminate the insoluble particles (millipore filter, 0.45 mm). The wines' samples were filtered through a 0.45 mm membrane before direct injection.

For preparation of the low pressure chromatography samples, 4 g of powder were ground for 2 min. in a blender with 10 mL of ethanol 950 mL and 10 mL aqueous solution containing 1 g/L of $NaHSO_3$ (antioxydizing agent). We added 20 mL of chloroform and mixed for an additional minite. The remaining solution was then centrifuged for 10 min. (x 4 000 g). Two phases were separated out by an interface constituated of the solid material. The green lower phase (containing chloroform, lipids, pigments...) was eliminated. The yellow superior phase (hydroalcoholic solution) containing the phenolic constituents was recovered. This extraction was repeated six times on the powder remaining in the tube of centrifugation. All the hydroalcoholic extracts were collected and were evaporated to remove ethanol (T ≤ 30 °C). The acqueous solution obtained was filtered. 20 mL of this solution or wine was extracted with ethyl acetate (6 x 20 mL). The organic phases were collected and the solvent was evaporated (T ≤ 30°C). The extracts obtained were soluble in 5 mL of methanol before being injected into a low pressure column.

The samples were injected into a low pressure column (1,6 x 35 cm) of gel TSK Toyopearl HW-40(S). They were eluted with methanol at a flow rate of 0,8 mL/min. The fractions containing the procyanidin oligomers were collected. The solvent was completely evaporated (T ≤ 30°C). The extracts were soluble in 0,5 mL of methanol before being analysed by HPLC.

IV - HPLC ANALYSIS

20 µL of the extract were injected onto two BECKMAN™ ultrasphere ODS C18 (250 x 46 mm; 5 µm) columns in series at 20 °C (± 1 °C), eluted with a flow rate of 1 mL/min. with the composition of two solvents: solvent A was formic acid: water (2.5:97.5, v/v), solvent B was solvent A: acetonitrile (20:80, v/v). The analytical method used was very similar to the proce-

dure described by RICARDO DA SILVA *et al.* (1991). The gradient conditions were :

temps (min)	0	5	90	95	100	105
% de A	93	93	80	0	0	93
% de B	7	7	20	100	100	7

Detection was monitored at O.D. 280 nm with a diode area detector. The levels of procyanidin dimers were quantified using standard curves developed from reference standards.

V - THIOLYSIS WITH TOLUEN-α-THIOL

The main compounds were collected by repeated injection and HPLC separation. The solvent were evaporated and the residue was dissolved in a sealed tube with 40 µL of toluen-α-thiol. The mixture was incubated at 100 °C in a bath-oil during 1 h to obtain the corresponding flavanol units in thioethers forms. After evaporation of toluene, we added 30 µL of RaNi (H_2 for 2 min.) in the dried extract and incubated at 50 °C during 1 h, for regenerated natif flavanols. The medium was then analysed by HPLC (Tr: catechin 29.5 min., epicatechin 48.6 min.).

VI - BATE-SMITH REACTION

Classical BATE-SMITH reaction (1972) were performed and the coloured compounds produced were isolated by liquid-liquid extraction with isoamylic alcohol.

VII - THRESHOLD DETERMINATION AND WINE TASTING

Procyanidins A2 and B2 threshold values was determined by a triangle tasting using a 17 people panel, all professional tasters and enologists. The value at 50 p. cent of the panel S_{50} were performed in hydro-alcoolic media with a composition similar than the one in wines (120 mL/L of ethanol, 5 g/L of tartaric acid, KOH N for pH 3.5), in a non-bitter and in a bitter wine. In addition all the wines of our experiments in order to evaluate the general qualities of the wines and especially the gustative aspect. The general quality level was evaluated by grades given the panel.

RESULTS AND DISCUSSION

I - IDENTIFICATION OF PROCYANIDINS A2 IN GRAPES AND WINES

Figure 2 present a characteristic chromatogram of the fraction containing procyanidins from grapes extract and wines. Identification of the main peaks was attempted using two procedures. Firstly we made a Bate-Smith reaction on the collected compounds to confirm the proanthocyanidin nature. Secondly, thiolysis and desul-

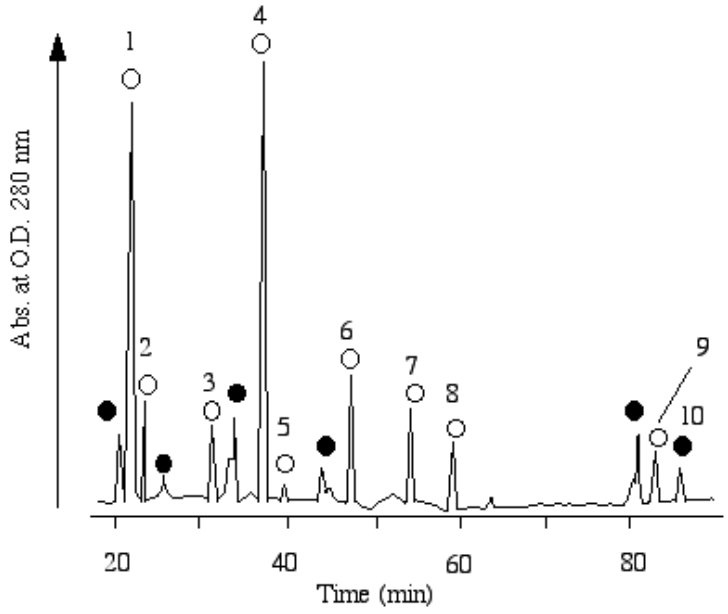

Fig. 1 - Chromatogram of reverse phase HPLC of a representative fraction of procyanidins from grapes seeds. Identification was performed by thiolysis with toluen-α-thiol. Full circles represent a positive thiolysis assay, empty circle represent a negative thiolysis assay (no flavanols were recovered aflter NiRa desulfuration). Identification: 1, B1; 2, B3; 3, B4; 4, B2; 5, B6; 6, B8; 7, unknow trimer; 8, B7; 9, B5; 10, A2.

Fig. 1 - Chromatogramme HPLC en phase interne d'une fraction représentative de procyanidoles issus de pépins de raisin. L'identification est réalisée par thiolyse au toluène-α-thiol. Les cercles pleins représentent un essai de thiolyse positif et les cercles vides représentent un essai de thiolyse négatif (pas de flavanols identifiés après désulfuration au NiRa).

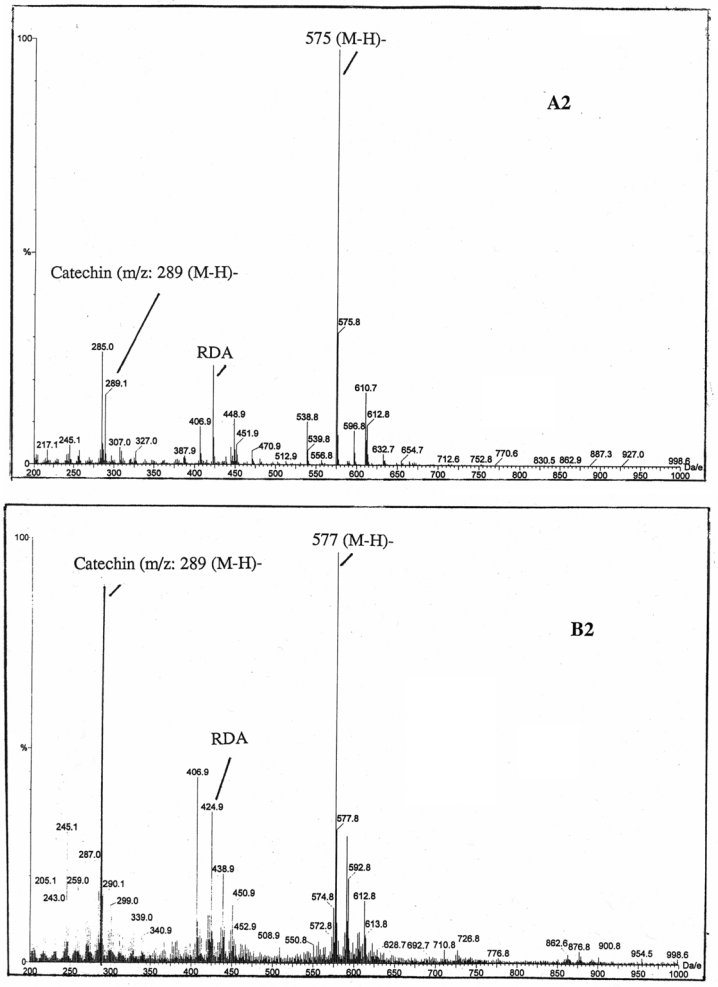

Fig. 2 - Mass spectrum of B2 and A2 procyanidins.

LC-MS experiment was realised by electrospray in negative mode at 100 V, after reverse phase HPLC fractionation. Spectra were realised at the top of each chromatographic peak, recorded by diode area detector at O.D. 280 nm. RDA, retro Diels-Alder.

Fig. 2 - Spectre de masse des procyanidoles B 2 et A2

L'expérience de LC-MS est réalisée par électrospray en mode négatif à 100 V, après séparation par HPLC en phase inverse. Les spectres sont réalisés au sommet de chaque pic chromatographique, enregistré par détecteur à barette diode à 280 nm. RDA, retro Diels-Alder.

TABLE 1
Concentration of procyanidins dimers A2 and B in grapes (seeds, skins) of *Vitis vinifera* L. Cabernet sauvignon and Merlot noir

(results are in mg/g of dried weight, average of 5 samples for each varieties)

Tableau I - Concentration des procyanidoles dimères A2 et B des les raisins (pellicules, pépins) de *Vitis vinifera L.* Cabernet sauvignon et Merlot noir

(Les résultats sont en mg/g de poids sec, moyenne de 5 échantillons pour chaque cépage)

Procyanidins	Merlot noir		Cabernet Sauvignon	
	Seeds	Skins	Seeds	skins
A2	4,7 ±1,8	3,2 ±1,4	5,7 ±2,1	3,1 ±1,3
B1	72,1 ±23,5	0,8 ±0,3	85,3 ±26,7	0,6 ±0,2
B2	188,3 ±31,5	8,9 ±2,6	258,1 ±56,4	4,7 ±1,5
B3	66,8 ±21,3	0,2 ±0,1	60,9 ±19,5	0,3 ±0,2
B4	104 ±17,5	0	115,6 ±20,2	0
B5	17,9 ±4,2	1,4 ±0,7	7,4 ±2,6	0,6 ±0,1
B6	24,6 ±5,7	0	18,7 ±4,9	0
B7	14,5 ±3,8	0	27,9 ±7,2	0
B8	12,1 ±2,1	0	13,6 ±1,3	0

TABLE II
Concentration of procyanidins dimers A2 and B in wines of *Vitis vinifera* L. Cabernet Sauvignon and Merlot noir
(results are in mg/L, average of 5 samples for each varieties)
Tableau II - Concentration des procyanidoles dimères A2 et B dans les vins issus de *Vitis vinifera* L. Cabernet Sauvignon et Merlot noir
(Les résultats sont exprimés en mg/L, moyenne de 5 échantillons)

Procyanidins	Merlot noir	Cabernet Sauvignon
A2	1 ±0,3	0,9 ±0,2
B1	21,6 ±3,2	10,9 ±2,7
B2	50,7 ±12,8	21,2 ±7,5
B3	8 ±2,4	4 ±1,3
B4	8 ±1,7	2,4 ±1,0
B5	9,5 ±2,6	6,4 ±1,9
B6	3,7 ±1,1	3,1 ±1,1
B7	2,6 ±1,1	1 ±0,3
B8	1,3 ±0,6	0,6 ±0,2

TABLE III
Procyanidins A2 and B2 threshold in hydroalcoolic solution and in different red wines
(1: hydroalcoolic solution; 2: non-bitter wine; 3: bitter wine; results are expressed in mg/l)
Tableau III - Seuil de perception des procyanidoles A2 and B2 en milieu hydroalcoolique et dans différents vins rouges
(1 : solution hydroalcoolique, 2 : vin non amer, 3 : vin amer ; les résultats sont exprimés en mg/l)

	Procyanidin A2			Procyanidin B2		
	1	2	3	1	2	3
Threshold[a]	4	26	1	35	nd	nd
Caracters:	0	0	ndc	+	nd	
Astringency[b]	+	+	nd	0	nd	
Bitterness[b]	+	+	nd	0	nd	

a) at 50% of the panel (S_{50}) - b) relative intensity (0, -, +) c) not determined

furation with RaNi; flavanols produced were detected by HPLC. On other hand we compared the retention time (Tr) and the UV spectra with diode area detectors of the unknown procyanidins. Concerning the B dimer series attributions were in accordance with the previous identifications (FREITAS, 1995) and all the LSIMS spectra gave a *m/z* 577 $(M-H)^-$. Concerning the peak at Tr 86.5 min., we confirm that it is a procyanidin by a Bate-Smith test, but after thiolysis, flavanol cannot be identified. In addition this compounds produce during Bate-Smith reaction 0.26 of cyanidins and 0.74 of a red product with the typical reaction of oxonium structure: variation of colour according to pH and decolorised by SO_2. The LSIMS spectra shows a principal peak at *m/z* 575 $(M-H)^-$. The Tr and UV spectra corresponding perfectly to pure A2. In fact, the product of the Bate-Smith reaction is in accordance with the results of JACQUES *et al.* (1974); this structure 1 provides a major oxonium with a maximum absorbance visible at 551 nm (in isoamylic alcohol). Ether linkage unpermitting the liberation of flavonols by thiolysis, we confirmed this by thiolysis experiments of pure A2; ether linkage additional bonded can explain this result.

Finally, the mass spectra experiments confirmed that it was a procyanidin dimer of A-series. These results permit to confirm the A2 presence in grapes and wines. A recent LC/MS experiment, with electrospray ionisation source, confirmed this attribution (figure 2).

II - QUANTIFICATION OF PROCYANIDINS A2. COMPARISON WITH OTHER PROCYANIDINS DIMERS OF B-SERIES

In tables I and II we resort the quantification, procyanidins B and A2. If some procyanidins B were localised only in parts of the grapes (B4, B6, B7, B8, only in seeds), A2 was identified with consistencies in all samples of skins and seeds. Grape seeds and skins present procyanidins A2 in equivalent quantities in Cabernet Sauvignon and in Merlot noir (5 mg/g in seeds, 3 mg/g in skins). All red wines analysed in this study show a concentration of A2 procyanidin near 1 mg/L. So this dimer is a very limited compound compared to B series in these varieties and in it is corresponding wines.

VI - THRESHOLD DETERMINATION (table III)

Bitter sensation of A2 was confirmed by these experiments. Also, in hydroalcoolic solution, A2 threshold is of 4 mg/L, near the threshold published by ROOYEN and REDELINGHUYS (1983). In the same condition, we found in B2 a threshold of 35 mg/L. So additional ether bonds are responsible for the decrease of gustative perception of the molecule and modifies it is tasty character. The low concentration of procyanidins A2 does not permit to influence bitterness of red wine. But A2 can give to non-bitter wines a typical bitterness.

CONCLUSION

In this study we have demonstrated the presence of procyanidins A2 in Cabernet sauvignon and Merlot noir grapes and wines. But this compound was found at a very limited concentration in grapes (5 to 3 mg/g respectively in seeds and skins) and wines (1 mg/L). At this concentration A2 is unable to participate to the bitterness of red wines. But the extensive knowledge

of the A series procyanidins in *Vitis vinifera* L. can permit to conclude definitively this hypothesis.

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